PHYSIOLOGY AND REPRODUCTION

An Effective Method for Improving the Fertility of Glycerol-Exposed Poultry Semen

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ABSTRACT Semen cryopreservation is necessary for banking germplasm from critical poultry stocks. To date, glycerol is the most effective cryoprotectant for poultry sperm; however, the contraceptive effects of glycerol require a significant reduction of the cryoprotectant from thawed semen before artificial insemination (AI). The effectiveness of glycerol reduction by dialysis, Percoll density gradient centrifugation, or washing through 12% (wt/vol) Accudenz was evaluated by fertility trials with highly inbred chicken research lines and commercial turkey lines. Semen was extended 1:1 and then diluted with glycerolized extender to yield a final 11% (vol/vol) glycerol concentration. Glycerolized rooster semen was aliquoted for control, Accudenz centrifugation, and dialysis treatments. A total of 90 pure line and 85 F_1 hybrid chicken hens were each inseminated with 100×10^6 sperm at 7-d intervals for 4 to 6 wk. All eggs from the glycerolized control semen treatments were infertile, and fertility rates from dialyzed semen decreased steadily from 26.4 to 0% within the first 4 wk for the pure lines. In contrast, fertility rates for Accudenz-processed semen increased from 17.9 to 37.17% during the first 4 wk. Similar fertility rates occurred with the F_1 hybrid cross lines. For turkey fertility trials, the dialysis treatment was not used; glycerolized turkey semen was processed by Accudenz or Percoll centrifugation to reduce glycerol. A total of 36 hens were inseminated with 150×10^6 sperm at 7-d intervals for 6 wk. Similar to the chicken trials, fertility rates of Accudenz-processed semen steadily increased to 49.4% by the sixth week of insemination. The average fertility of Percoll-processed semen was only 19.1%. These data demonstrate that Accudenz centrifugation is an acceptable glycerol reduction method for nonfrozen poultry semen.

(*Key words*: turkey, rooster, semen, glycerol, Accudenz)

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INTRODUCTION

The ability to cryopreserve and store germplasm has long been valued for the indefinite preservation of genetic material, especially for at-risk populations. In particular, there is an immediate need for storing germplasm from unique research poultry lines. A growing percentage (>37%) of the genetically diverse poultry stocks developed by academic researchers have disappeared or become at-risk in recent years (Pisenti et al., 1999). For example, in 1997, a genetically significant poultry collection comprising 72 specialty and historical commercial stocks located at the Center for Farm Animal Research in Ottawa, Ontario was almost eliminated due to loss of funding support. It is generally agreed that cryopreserved poultry semen could be utilized for germline retrieval (L. D. Bacon, personal communication; Tajima et al., 1990); however, fertility rates from frozen/thawed poultry semen using current cryogenic protocols are not adequate to recover poultry stocks.

Although it is evident that fertility rates for germline retrieval can be much lower (e.g., 20 to 40%) than that required for commercial production (>90%), cryopreserved sperm must retain sufficient functionality to avoid using excessively high insemination doses ($>500 \times 10^6$ sperm) and frequent inseminations (3 to 4 times/wk). Because several generations will be required to recover germlines using sperm as the only gamete source, prudent usage of stored, cryopreserved semen will be necessary to avoid depleting the stockpile. Most literature reports of high fertility rates with frozen/thawed semen used these accentuated insemination schemes (Lake et al., 1981; Kurbatov et al., 1988; Bellagamba et al., 1993; Gill et al., 1996). One exception is the use of the cryoprotectant dimethylacetamide in conjunction with the pellet method of freezing directly in liquid nitrogen (Tselutin et al., 1999). However, straw packaging is recommended for gene banking to ensure high levels of safety and clear

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Abbreviation key: ADOL = Avian Disease and Oncology Laboratory; AI = artificial insemination; BPSE = Beltsville poultry semen extender.

identification, and very low fertility rates were obtained when DMA was used in conjunction with straws (Tselutin et al., 1999).

Of the cryoprotectants studied to date, glycerol appears to be the most effective for protecting poultry sperm during the cryogenic cycle (Maeda et al., 1984; Bacon et al., 1986; Hammerstedt and Graham, 1992; Donoghue and Wishart, 2000). Concentrations of glycerol needed to provide adequate protection (~1 *M*) are contraceptive in the hen and must be lowered to <0.1 *M* before insemination of thawed semen (Neville et al., 1971; Sexton, 1973a, 1975; Phillips et al., 1996). The precise reason for the lowered fertility of sperm inseminated in the presence of glycerol is unknown, but is likely related to the osmotic shock following rapid loss of glycerol from sperm in the hen's reproductive tract and subsequent disruption of the cell membrane (Westfall and Howarth, 1978; Lake et al., 1980).

It is well known that glycerolized sperm do not populate the sperm storage tubules. For example, histological sections revealed the absence of sperm in the tubules after intravaginal insemination with glycerolized turkey sperm (Marquez and Ogasawara, 1977a). Glycerol is believed to adversely affect the sperm cells rather than the tubule's ability to store sperm, as timed inseminations of glycerol before, during, and after insemination resulted in the greatest contraceptive effect when glycerol and sperm were mixed (Westfall and Howarth, 1977a).

Researchers have examined the feasibility of bypassing sperm storage in the tubules by using intramagnal insemination and achieved 53 to 70% fertility with glycerolized chicken (Bacon et al., 1986; Salter et al., 1987) and turkey (Marquez and Ogasawara, 1977a) semen. Intramagnal insemination is a surgical procedure that may disrupt egg lay in the female and should not be conducted multiple times on the same hen; therefore, it is not advisable to use this technique alone to circumvent the contraceptive effect of glycerol for germline retrieval. Methods of removing glycerol from semen before insemination have been explored with varying success (Lake and Stewart, 1978; Lake et al., 1981; Buss, 1993; Phillips et al., 1996). For example, glycerol reduction has been achieved by serial dilution of chicken semen with glycerol-free media and concentration of the sperm with centrifugation; however, even with a large inseminant dose (2×10^8 sperm), fertility rates were less than 21% (Yousif et al., 1984).

It is clear that the potential loss of current poultry stocks in the near future may precede development of alternate cryopreservation protocols for poultry semen. In that context, we present findings from our initial investigation of glycerol reduction methods for nonfrozen chicken and turkey sperm. In particular, we used unique inbred chicken lines from the USDA Avian Disease and Oncology Laboratory (ADOL) to demonstrate the applicability for preserving typical research stocks. The ADOL lines were initiated in 1939 for the study of Marek's disease resistance and immune response traits, and are 99% inbred. Because the method described here was successful with nonfrozen sperm, we anticipate that further development of this glycerol reduction protocol using frozen/thawed sperm will provide the means for immediate storage of semen from at-risk poultry stocks and will enable germline recovery from semen frozen with glycerol as the cryoprotectant.

MATERIALS AND METHODS

Chicken Management

Two populations were used for this study: ADOL pure lines $15I_5$ and 7_1, and an F_1 hybrid cross ($15I_5$ males × 7_1 females). The pure lines were produced and used at ADOL; the fertile F_1 hybrid cross eggs were shipped to the Beltsville ARS location, where chicks were hatched, grown, and maintained using the same husbandry practices as for the ADOL pure lines. Pure line and F_1 hybrid cross birds were exposed to 16L:8D. Pure line roosters and hens were 84 wk of age when the study was initiated. Pure line hens had not been inseminated for 8 wk before the study, and eggs produced by these hens were infertile for 3 wk before initiation of the study. The F_1 hybrid birds were 34 wk of age and had not been inseminated before initiation of the study.

Turkey Management

Large White breeder poults were purchased from a primary breeder[2] and maintained under standard management conditions during the brooding and growing periods. At 28 wk of age, toms were photostimulated by increasing light exposure from 12L:12D to a 14L:10D cycle to initiate semen production. At 32 wk of age, hens were photostimulated by increasing the daily light exposure from 6L:18D to 14L:10D to initiate ovarian development and egg production. Toms and hens were 48 wk of age when the experiment was initiated; turkey hens had not been inseminated with semen prior to this study.

Semen Collection, Processing, and Glycerol Exposure

Using the abdominal massage method (Burrows and Quinn, 1937), semen was collected at 7-d intervals from 10 pure line (ADOL location) and 14 F_1 hybrid cross (BARC location) roosters. Semen also was collected by abdominal massage from 10 toms at 7-d intervals.

Semen samples were pooled by line or species and evaluated for total volume, sperm concentration, sperm viability, and sperm mobility before 1:1 dilution with Lake's extender (chicken) or Beltsville Poultry Semen Extender[3] (BPSE; turkey). Extended semen was diluted further with Lake's extender or BPSE containing 33% (vol/vol) glycerol to obtain a final concentration of 11% glycerol. This glycerol concentration is considered the optimal concentration for freezing poultry sperm (Westfall and

[2]British United Turkeys of America, Lewisburg, WV.
[3]Continental Plastics Corp., Delavan, WI.

Harris, 1975; Lake et al., 1980; Hammerstedt and Graham, 1992; Seigneurin and Blesbois, 1995; Tselutin et al., 1999). For chicken semen, additions of glycerol were conducted at room temperature or after cooling to 4°C for 30 min (see Experimental Design below). The glycerolized diluent was added slowly in a drop-wise fashion with intermittent finger-vortex mixing. After 30 min equilibration at room temperature or 4°C, glycerolized semen was divided into aliquots for control (glycerol not removed) and glycerol reduction methods as detailed below. For chicken and turkey semen exposed to glycerol at room temperature, a maximum of 1 h elapsed between the time of semen collection and artificial insemination (AI); whereas a maximum of 1.5 h elapsed between the time of semen collection and AI for chicken semen that was cooled prior to the addition of glycerolized cryodiluent.

Glycerol Reduction Methods

Dialysis. Slide-A-Lyzer[4] dialysis cassettes with a molecular weight cutoff of 10,000 and a 1-mL volume capacity were used. The cassette membrane contained 12% glycerol. After hydration, cassettes were loaded with 1 mL of glycerolized semen and immersed in 400 mL of the appropriate glycerol-free extender (Lake's or BPSE) at room temperature for 30 min. During dialysis, glycerol-free extender was gently agitated on a stir plate. In preliminary tests, dialysis times of 10, 20, 30, 60, 120, and 240 min were evaluated, as well as temperatures of 4 and 25°C. Data indicated that 30 min at room temperature was optimal for recovery of motile sperm.

Accudenz Gradient Centrifugation. The semen washing method developed by McLean et al. (1998) as a tool for evaluating the effects of specific chemicals on sperm function was investigated as a means of glycerol reduction. Discontinuous gradients were prepared in 15-mL conical centrifuge tubes by depositing 0.5 mL of 30% (wt/vol) Accudenz[5] under a 5-mL volume of 12% (wt/vol) Accudenz, using the stock solution preparations previously described (McLean et al., 1998). A maximum of 1 mL of glycerolized semen was gently layered on the discontinuous gradient and tubes were centrifuged (1,250 × *g*; 4°C) for 25 min with a gradual stop (e.g., without using the brake). After centrifugation, the extender and seminal plasma remained above the 12% layer, whereas sperm cells were present at the interface between the 12 and 30% layers. On occasion, a pellet was observed at the bottom of the tube that contained poor or nonmotile sperm and particulate debris. To recover the sperm layer, the upper gradient layer was removed first. The 30% layer was then aspirated, using a new pipette tip, leaving the sperm layer in the centrifuge tube. Because of the viscosity of the remaining sperm layer, up to 1 mL of extender was added (depending on the volume of the original semen layer) and mixed before recovering the sperm layer.

Percoll Density Gradient Centrifugation. Commercially prepared 40 and 80% Percoll solutions[6] were used for the discontinuous gradients, with the lower phase as the higher density. Glycerolized semen (1 mL maximum) was carefully layered on the upper phase for a total volume of 11 mL in a 15-mL conical tube. Gradients were centrifuged (400 × *g*; room temperature) for 20 min. After centrifugation, the upper and lower phases were aspirated from the sperm pellet and the pellet was washed by resuspending in 5 mL of the appropriate extender and centrifuging (200 × g; 10 min). After removing the supernatant, the pellet was resuspended with up to 1 mL of extender (depending on the volume of the original semen layer).

Sperm Concentration, Membrane Viability, and Mobility Assays

Sperm concentration was determined using an IMV Microreader photometer[7] to estimate the optical density of a 1:200 dilution of neat semen in a 3% (wt/vol) Na citrate solution. Sperm viability was determined using the SYBR-14/propidium iodide live/dead stain combination as evaluated by flow cytometry (Donoghue et al., 1995). Sperm mobility was assessed as previously described (Froman and McLean, 1996) with minor modifications (Long et al., 2003). In brief, a sperm suspension (1×10^9 sperm/mL of mobility buffer) was overlaid onto a 6% Accudenz solution and incubated for 5 min at 41°C. The optical density was measured by an IMV Microreader photometer (OD 597) after 1 min of equilibration; sperm mobility values are presented as absorbance units[4]. It has been demonstrated that the frequency of sperm mobility values for individual males approximates a normal distribution (Froman and Feltmann, 1998). Therefore, individual male sperm mobility values that are one standard deviation above or below the population mean are categorized as high or low mobility males, respectively. In general, poultry sperm mobility values above 0.6 are considered high, whereas sperm mobility values below 0.2 are classified as low (Froman and Feltmann, 1998; King and Donoghue, 2000).

Fertility and Hatchability Evaluations

To determine chicken fertility and hatchability rates, 90 pure line and 85 F_1 hybrid hens were each inseminated with 100×10^6 sperm at 7-d intervals for 4 to 6 wk. For turkey fertility and hatchability evaluations, 36 hens were inseminated with 150×10^6 sperm at 7-d intervals for 6 wk. The sperm doses used were lower than those typically used for commercial production because we desired a system sufficiently sensitive to detect even small changes in fertility due to semen processing treatment. After 7 d of incubation, eggs were candled to determine fertility.

[4]Pierce Biotechnology, Rockford, IL.
[5]Accudenz®, Accurate Chemical & Scientific Corporation, Westbury, NY.
[6]Sigma-Aldrich, St. Louis, MO.
[7]IMV, Minneapolis, MN.

Hatchability was determined after 21 and 28 d of incubation, respectively, for chicken and turkey eggs.

Experimental Design

Two trials were conducted to compare the dialysis and Accudenz centrifugation methods of glycerol removal from rooster semen. The trials were similar except for bird strain and glycerol equilibration temperature. In trial 1, the experiment was conducted with ADOL pure lines $15I_5$ (male) and 7_1 (female) at East Lansing, MI and all semen processing was conducted at room temperature. Trial 2 was conducted at the Beltsville ARS location with an F_1 hybrid cross ($15I_5$ males $\times$ 7_1 females) and semen was cooled to 4°C before addition of precooled (4°C) glycerol. In trial 1, pure line hens (control or glycerolized semen, n = 29 hens; dialysis, n = 30 hens; Accudenz gradient, n = 31 hens) were inseminated with pooled semen from pure line roosters. In trial 2, F_1 hybrid hens (control or nonglycerolized semen, n = 27 hens; dialysis, n = 29 hens; Accudenz gradient, n = 29 hens) were inseminated with pooled semen from F_1 hybrid roosters. The sperm concentration, viability, and mobility of semen were re-evaluated after glycerol removal. Inseminant volumes for dialysis and Accudenz-processed semen were adjusted to correct for losses in sperm number. As a result, the inseminant volumes for dialyzed semen ranged from 80 to 125 μL, and the inseminant volume range of Accudenz-processed semen (12 to 35 μL) was similar to the control (10 to 25 μL).

Based on the results of the rooster semen trials, 2 gradient centrifugation protocols (Accudenz and Percoll) were evaluated as methods to remove glycerol from turkey semen. Twelve hens per semen treatment (control or nonglycerolized semen; Accudenz gradient; Percoll gradient) were inseminated with pooled semen from 10 toms. As in the rooster trials, the concentration, viability, and mobility of turkey semen were re-evaluated after glycerol removal and inseminant volumes corrected for losses in sperm number, with AI volume ranges of 25 to 35 μL for control and Accudenz-processed semen, and 75 to 120 μL for Percoll-processed semen.

Statistical Analysis

The following data were subjected to statistical analysis: number of sperm recovered after glycerol removal, percentage of membrane intact sperm, sperm mobility score, percentage of fertile eggs, and percentage of hatched eggs. Before analysis, data were checked to determine if a transformation was necessary. As expected, variables scored as percentages (sperm viability, fertility, hatchability) required transformation. We used the standard variance stabilizing arcsine transformation, y = arcsine ($\sqrt{(x)}$), where x represents the data, recorded as a proportion, and y is the scale used in the analysis. This transformation stabilized the variances sufficiently to proceed with an analysis in a mixed models framework. The mixed models (Proc Mixed, SAS Institute, 1999) considered sperm treatment and week of study as fixed factors, and hen as a random factor. For modeling fertility, we assumed the variance on the transformed scale due to sampling eggs was 0.25/n, where n is the number of eggs/hen per wk (Steel and Torrie, 1960).

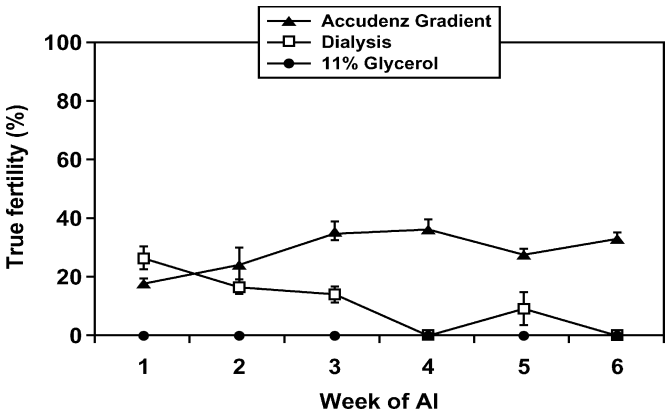

FIGURE 1. Fertility (± SEM) of glycerolized rooster semen after glycerol reduction by Accudenz centrifugation or dialysis, compared with control semen containing 11% glycerol. Pure line 7_1 hens (84 wk old) were inseminated at 7-d intervals with 100×10^6 sperm for 6 wk. Fertility rates were higher ($P < 0.05$) for Accudenz-processed than control semen during the 6-wk insemination period. The fertility rates of dialyzed semen were higher ($P < 0.05$) than control semen only for the first 3 wk of insemination. AI = artificial insemination.

RESULTS

Glycerol Reduction: Dialysis Vs. Accudenz Gradient Centrifugation

For ADOL pure line and F_1 hybrid cross trials, the overall fertility of Accudenz-processed semen was higher ($P < 0.05$) than dialysis-processed semen (Figures 1 and 2). In both trials, the fertility of dialyzed semen decreased

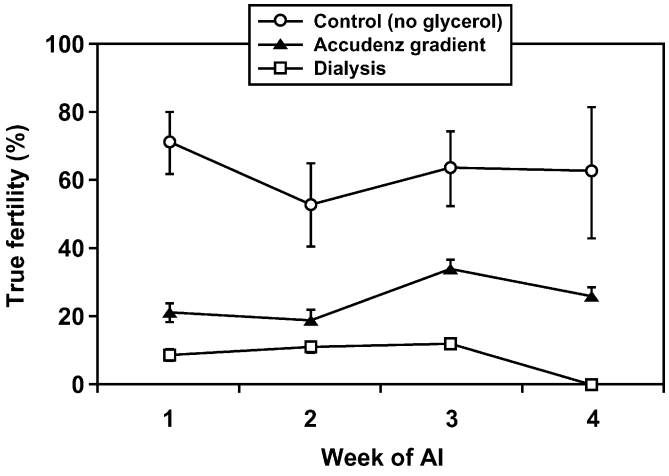

FIGURE 2. Fertility (± SEM) of glycerolized rooster semen after glycerol reduction by Accudenz centrifugation or dialysis, compared with the nonglycerolized, control semen. F_1 hybrid cross hens (34 wk old) were inseminated at 7-d intervals with 100×10^6 sperm for 4 wk. Fertility rates were higher ($P < 0.05$) for Accudenz-processed than dialyzed semen during the 4-wk insemination period. AI = artificial insemination.

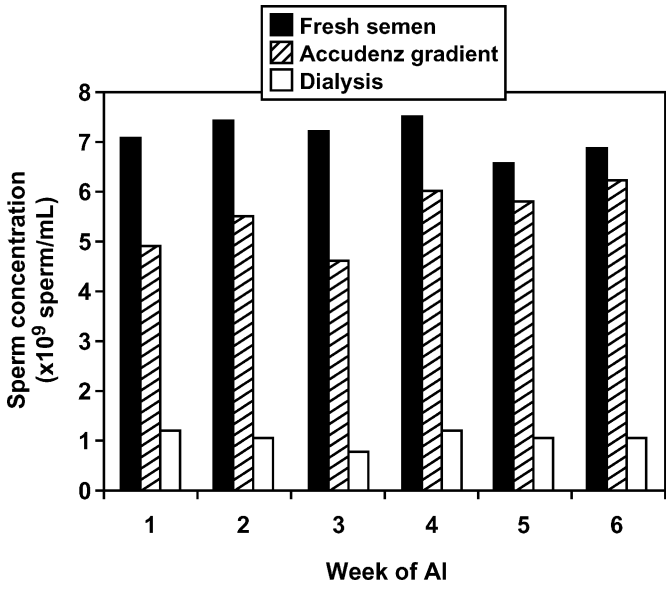

FIGURE 3. Rooster sperm recovery after glycerol reduction by Accudenz centrifugation or dialysis, compared with the nonprocessed, glycerolized semen aliquot. Fewer sperm were recovered from the dialysis treatment than from the Accudenz centrifugation ($P < 0.05$). Inseminant dose volumes were adjusted to obtain 100×10^6 sperm, resulting in larger volumes for hens inseminated with dialyzed (80 to 125 μL) than Accudenz-processed (12 to 35 μL) or control (10 to 25 μL) semen. AI = artificial insemination.

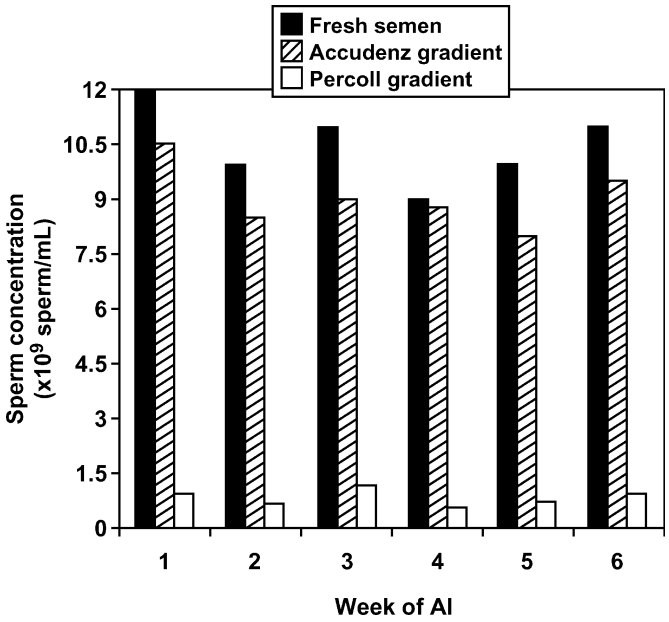

FIGURE 5. Turkey sperm recovery after glycerol reduction by Accudenz or Percoll density gradient centrifugation, compared with the nonprocessed, fresh semen aliquot. Fewer sperm were recovered from the Percoll than the Accudenz gradient ($P < 0.05$). Inseminant dose volumes were adjusted to obtain 150×10^6 sperm, resulting in larger volumes for hens inseminated with Percoll-processed (75 to 120 μL) than Accudenz-processed (25 to 35 μL) semen. AI = artificial insemination.

steadily during the 4 to 6 wk insemination period, from 26.4 ± 3.9 to 0% for the pure line birds and from 16.2 ± 2.1 to 3.1 ± 0.2% for the F_1 hybrid birds. In contrast, the fertility of Accudenz-processed semen was lowest during wk 1 (17.9 ± 1.2%, pure line; 20.9 ± 1.6%, F_1 hybrid) and 2 (24.1 ± 1.1% pure line; 19.4 ± 2.1% F_1 hybrid) of insemination, and peaked during wk 3 (34.3 ± 2.7%) and 4 (37.1 ± 3.3%) for the F_1 hybrid cross and pure lines, respectively. No fertile eggs were obtained from pure line hens when glycerolized semen was inseminated. It should be noted that the average fertility of fresh semen (not exposed to glycerol) during the 6-wk insemination period was 62% for the F_1 hybrid cross (Figure 2); whereas the pure line hens averaged 77% fertility before initiation of the study (data not shown). Additionally, no differences ($P > 0.05$) were detected in the hatchability of fertile eggs, regardless of bird line or semen treatment (range 82.7 to 84.5%).

The total number of sperm recovered after glycerol reduction was lower ($P < 0.05$) for dialyzed semen than for Accudenz-processed semen for the pure line (Figure 3) and F_1 hybrid cross (data not shown) birds. Average sperm viability (assessed for F_1 hybrid semen only) was higher ($P < 0.05$) for fresh (93.3%) than for glycerol-exposed (82.8%) semen; no differences ($P > 0.05$) in the percentage of viable sperm were observed between dialyzed (81.3%) and Accudenz-processed (83.4%) semen. In contrast, the average sperm mobility (assessed for F_1 hybrid semen only) was higher ($P < 0.05$) for Accudenz-processed (0.178 ± 0.024) than dialyzed (0.046 ± 0.014) semen. Pretreatment sperm mobility values for the hybrid Leghorn males in this study (mean, 0.203 ± 0.06) were lower than that observed for commercial broiler males in recent experimental trials (range, 0.261 to 0.526; J. A. Long, unpublished data).

Glycerol Reduction: Accudenz Vs. Percoll Density Gradient

The overall fertility of turkey semen processed with the Accudenz gradient was higher ($P < 0.05$) than with the Percoll gradient (Figure 4). Similar to the findings in

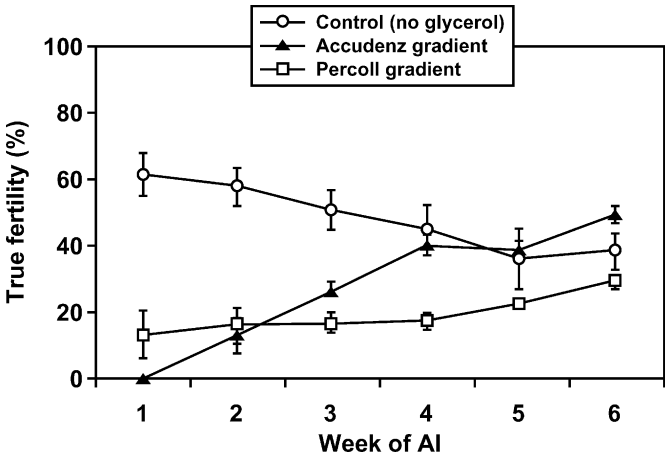

FIGURE 4. Fertility of glycerolized turkey semen after glycerol reduction by Accudenz or Percoll density gradient centrifugation, compared with the nonglycerolized, control semen. Turkey hens (48 wk old) were inseminated at 7-d intervals with 150×10^6 sperm for 6 wk. Fertility rates were higher ($P < 0.05$) for Accudenz-processed than Percoll-processed semen during wk 3 to 6 of the study. AI = artificial insemination.

the chicken fertility trials, initial fertility rates were low for the Accudenz-processed semen; in fact, no fertile eggs were obtained from the first insemination. During the subsequent 5-wk insemination period, however, fertility rates increased to 57.6%. In contrast, the fertility rates for the Percoll gradient remained low (range, 13.3 to 17.5%) during the first 4 wk of insemination, and increased to only 23.8% by the sixth week of insemination. Interestingly, the fertility of fresh, nonglycerolized turkey semen gradually diminished during the insemination period, from 77.0 to 38.1%. Average hatchability rates were high and similar ($P > 0.05$) for all 3 groups (range, 93.5 to 100%).

The total number of sperm recovered after glycerol reduction was significantly lower ($P < 0.05$) for Percoll-processed than for Accudenz-processed turkey semen (Figure 5). Interestingly, the average sperm viability was higher ($P < 0.05$) for both gradient-processed semen treatments (92.1, Accudenz; 94.5%, Percoll) than for fresh, nonglycerolized semen (83%). The average sperm mobility was higher ($P < 0.05$) for fresh (0.203 ± 0.027) and Accudenz-processed (0.209 ± 0.05) than for Percoll-processed (0.137 ± 0.003) semen.

DISCUSSION

We present a new glycerol reduction method to enable successful germline retrieval from cryopreserved poultry semen. Because glycerol is contraceptive at concentrations >0.1 *M* (Hammerstedt and Graham, 1992), the success of a glycerol reduction method can be verified by fertility trials. As a first step in developing a glycerol reduction method suitable for frozen/thawed poultry sperm, we evaluated the fertility rates of nonfrozen spermatozoa that were equilibrated in a cryodiluent containing glycerol. In our study, glycerolized, fresh semen processed by Accudenz centrifugation yielded fertility rates of 36 and 50% in the chicken and turkey, respectively. These fertility rates are significant for several reasons. First, the fertility results were achieved using relatively low semen doses, ranging from 100 to 150 $\times 10^6$ sperm. It follows that increasing the insemination dose would provide a greater number of fertile eggs without using excessive amounts of a finite, stored semen supply. Second, the chicken fertility rates were achieved with highly inbred research lines as the experimental model, thus approximating an actual germline retrieval scenario. It is well known that the fertility of inbred lines is lower than commercial lines, and the fertility rates of nonglycerolized semen from the ADOL lines averaged 62%, compared with >90% fertility typically obtained with outbred Leghorn strains. Finally, it should be noted that germline retrieval can be accomplished with fertility rates ranging from 20 to 40%, as long as the hatchability of fertile eggs is high. In the present study, hatchability rates were greater than 80 and 90% respectively, for chicken and turkey insemination trials.

In our investigation, we first compared dialysis and Accudenz centrifugation as glycerol reduction methods for glycerolized semen from highly inbred Leghorn-type chickens. Simple dialysis of poultry semen has been used to improve the fertility of rooster semen held for 24 h at 4°C (Graham and Shangren, 1986; Blesbois and de Reviers, 1992), and to prepare semen for cryopreservation with cryoprotectants other than glycerol (Van Voorst and Leenstra, 1995). The only report of dialysis as a glycerol reduction method involved a patented Biostore environmental control chamber,[8] which required the use of a specialized freezing container (Buss, 1993) rather than straw packaging. In our study, glycerolized semen processed by Accudenz centrifugation provided more fertile eggs than dialysis. One major difference between the 2 glycerol reduction methods was that the number of sperm recovered after dialysis was considerably lower than that for gradient centrifugation. It is possible that significant numbers of sperm were lysed or adhered to the dialysis membrane. Because inseminant volumes were adjusted to provide a consistent number of sperm for each insemination, the final volumes of dialyzed semen were 2- to 3-fold higher than gradient-processed semen. The relatively low fertility rates achieved with dialyzed semen may also indicate that glycerol reduction was insufficient using this method.

Comparison of the 2 centrifugation methods demonstrated that the Accudenz protocol was superior to the Percoll protocol in the ability to reduce the level of glycerol. We speculate that although the Percoll method may have sufficiently lowered the glycerol concentration, the actual centrifugation procedure was harmful to sperm function. With the Percoll method, a sperm pellet is formed at the bottom of the centrifuge tube, which is then resuspended and centrifuged a second time to remove traces of the gradient material. Repeated centrifugation has been shown to be harmful to chicken sperm (Sexton, 1973b) and low-speed centrifugation of turkey sperm to remove glycerol resulted in loose acrosomes and ruptured cells (Marquez and Ogasawara, 1977b). With the Accudenz method, the sperm are cushioned by the lower layer rather than being pelleted against the wall of the centrifuge tube. Additionally, because Accudenz is an inert, nontoxic substance (Froman and McLean, 1996), a wash step is not required before insemination.

Although no fertile turkey eggs were obtained from the first AI with Accudenz-processed semen, it should be emphasized that the turkey hens were inseminated relatively late in the production period with semen from toms of the same age. It is an established practice for commercial turkey production to initiate inseminations 7 to 14 d before egg production in order to maintain persistent fertility levels (McIntyre et al., 1982; McIntyre and Christensen, 1985; Bakst, 1988). In the current study, the inseminations in late production likely contributed to the poor fertility rates associated with the first week of insemination, and to the gradual decline in fertility during the 6-wk trial. Given the age of the semen donors, it was not surprising that turkey sperm viability was improved

[8]BioPore, Inc., Centre Hall, PA.

after centrifugation, as labile spermatozoa present in fresh semen most likely were physically removed or lysed.

The successful glycerol reduction method presented here does not alleviate the need for developing alternative cryoprotectants for poultry semen. On the contrary, it has been demonstrated that simply exposing poultry sperm to glycerol causes damage (Latorre et al., 1988; Delee et al., 1991; Chalah et al., 1999) that may not be reversed upon glycerol removal. For example, after a 30 min exposure of rooster sperm to 15% glycerol, sperm potassium and magnesium concentrations were lowered and calcium levels were 2.5 fold higher than control sperm; neither centrifugation nor dialysis restored cation concentrations to normal values (Westfall and Howarth, 1977b). Additionally, glycerol can be metabolized by avian sperm to produce methylglyoxal, which in high concentrations (>60 μM) is a potent inhibitor of oxidative respiration (Riddle and Lorenz, 1973).

Because glycerol exposure alone has deleterious effects on sperm function, we evaluated the merits of glycerol reduction methods using fresh semen. We recognize that the Accudenz glycerol reduction method must be evaluated with frozen/thawed poultry semen before being incorporated into thawing protocols. Experiments are underway to evaluate the fertility of frozen/thawed semen from ADOL lines, using commercial hens as the hen source. As hens from a banked research line most likely would not be available (e.g., in live production) for insemination, this experimental model represents a more accurate line retrieval scenario.

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